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Multilocus Sequence Typing Reveals Intrafamilial Transmission and Microevolutions of *Candida albicans* Isolates from the Human Digestive Tract

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***Candida albicans* is a human commensal that is also responsible for superficial and systemic infections. Little is known about the carriage of *C. albicans* in the digestive tract and the genome dynamics that occur during commensalisms of this diploid species. The aim of this study was to evaluate the prevalence, diversity, and genetic relationships among *C. albicans* isolates recovered during natural colonization of the digestive tract of humans, with emphasis on Crohn's disease patients who produce anti-yeast antibodies and may have altered *Candida* sp. carriage. *Candida* sp. isolates were recovered from 234 subjects within 25 families with multiple cases of Crohn's disease and 10 control families, sampled at the oral and fecal sites. Prevalences of *Candida* sp. and *C. albicans* carriage were 53.4% and 46.5%, respectively, indicating frequent commensal carriage. No differences in prevalence of carriage could be observed between Crohn's disease patients and healthy subjects. Multilocus sequence typing (MLST) of *C. albicans* isolates revealed frequent colonization of a subject or several members of the same family by genetically indistinguishable or genetically close isolates. These latter isolates differed by loss-of-heterozygosity events at one or several of the MLST loci. These loss-of-heterozygosity events could be due to either chromosome loss followed by duplication or large mitotic recombination events between complementary chromosomes. This study was the first to jointly assess commensal carriage of *C. albicans*, intrafamilial transmission, and microevolution. The high frequency of each of these events suggests that the digestive tract provides an important and natural niche for microevolutions of diploid *C. albicans* through the loss of heterozygosity.**

Candida albicans is currently the most important opportunistic fungal pathogen of humans, responsible for both superficial and systemic infections (7). Clinical manifestations of *C. albicans* infections include superficial candidiasis infections (cutaneous candidiasis, oropharyngeal candidiasis, and vulvovaginitis) that are frequent but usually benign in immunocompetent hosts. They also include severe infections in hospitalized patients, in particular, candidemia and disseminated candidiasis, that are associated with high mortality rates. These systemic infections occur in numerous patients with severe underlying diseases or critical illnesses that need aggressive diagnosis or treatment procedures. As a consequence, *C. albicans* is the leading cause of nosocomial fungal infections (7).

Despite being a fungal pathogen, *C. albicans* is carried without symptoms by a large fraction of the population. Indeed, *C. albicans* colonizes mucosal surfaces of healthy subjects and is considered to be a component of the normal digestive and genital floras. In this regard, intestinal colonization is recognized as a key component of further development of both superficial and systemic *C. albicans* infections (8, 9, 24). However, frequency and chronology of carriage are only partially known, and much of the biology of *C. albicans* in the commensal stage remains to be understood. Early molecular epidemiological studies have shown that healthy subjects can be colonized simultaneously or sequentially by different strains of *C. albicans*, indicating that carriage is a dynamic process (46). During the life of an individual, *C. albicans* isolates can persist, evolve through minor genetic variations (referred to as microevolutions), or be replaced by other isolates (16–18).

The genetic mechanisms that underlie these microevolutions and their role in the diversification of *C. albicans* populations and in the adaptive response to different host environments have not been investigated. *C. albicans* is a diploid organism that has no known full sexual cycle (2). Several studies indicate that complementary chromosomes show a high level of allelism, and mitotic recombinations between complementary chromosomes are a probable source of genetic microevolutions (10, 11, 15, 47). In addition, the *C. albicans* genome contains genes that are homologous to those necessary for mating and meiosis in the yeast *Saccharomyces cerevisiae*, and it has been shown that mating-type-compatible *C. albicans*

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diploid strains are able to mate and form tetraploids (13, 19, 43). However, meiotic divisions have not been observed. Instead, tetraploids can contribute a parasexual cycle yielding diploid progenies (1). This parasexual cycle may represent a source of microevolutions through chromosome reassortments due to random distribution and/or mitotic recombination.

Molecular typing methods provide insights into the genetic diversity of a species as well as the mechanisms underlying the acquisition of such diversity. Molecular epidemiology studies, mainly carried out through fingerprinting of genomic DNA with the mildly repetitive sequence Ca3, have revealed that commensal and infecting isolates of *C. albicans* exhibit a high level of genetic diversity and are distributed within five major genetic groups, or clades (namely, clades I, II, III, E, and SA), with different geographic distributions (3, 28, 30). However, fingerprinting with the Ca3 probe does not provide the resolution necessary to infer the mechanisms that are at the origin of genetic microevolutions. In contrast, multilocus sequence typing (MLST) can be used for the overall analysis of the *C. albicans* population and for the detection of minute genetic changes that are indicative of evolutionary processes. MLST of *C. albicans* is a highly discriminatory method based on the analysis of nucleotide polymorphisms within the sequences of six or seven PCR-generated 400- to 500-bp internal fragments of housekeeping genes (loci) (5, 6). For each locus, the different sequences are assigned as distinct genotypes (represented by integers), and for each isolate, the combination of the genotypes at each of the sequenced loci defines a profile referred to as a diploid sequence type (DST). Because MLST relies only on nucleotide sequencing, it generates highly standardized data that can be exchanged through a web-based database (http://calbicans.mlst.net) (4). Importantly, it has been shown by us and others that MLST groups *C. albicans* strains into clades that are superimposable with those revealed by Ca3 fingerprinting, although analysis of large strain collections suggests the occurrence of new clades (39). Furthermore, MLST provides nucleotide sequence data that can be used to understand the genetic processes that underlie the evolutionary dynamics of strains within the overall population and within clades (40) (M. E. Bougnoux, C. Pujol, S. Morand, C. Bouchier, D. Soll and C. d'Enfert, Abstr. 7th ASM Conf. Candida Candidiasis, abstr. 72, 2004).

The human digestive tract is probably among the most relevant sources to investigate the natural history of these evolutionary processes. In this regard, in the present study, we have taken advantage of a collection of *C. albicans* strains obtained as part of a large epidemiological study of patients with Crohn's disease (CD) and their families (44). CD is a severe inflammatory bowel disease whose etiology is poorly understood. Predisposition to CD is thought to combine genetic and environmental/endoluminal factors (26). Among these factors, it has been established that 50 to 70% of the CD patients produce anti-yeast antibodies; these are antibodies reacting with *S. cerevisiae* mannan and are named anti-*Saccharomyces cerevisiae* antibodies (ASCA) (31, 35). ASCA-positive subjects represent only 5% of the control population (27), while they are found in 20% of relatives of CD patients (37). In patients, ASCA are associated with severe ileal forms and are used for differential diagnosis/stratification (22, 45). The endogenous immunogen for ASCA is unknown, but recent clinical and experimental evidence has shown the production of such an immunogen by *C. albicans* under certain growth conditions including pathogenic development (A. Standaert-Vitse, T. Jouault, P. Vandewalle, C. Mille, M. Seddik, B. Sendid, J. M. Mallet, J. F. Colombel, and D. Poulain, unpublished data). Mycological sampling was therefore included in the abovementioned study (44) in order to test the hypothesis that *C. albicans* could be one component of the human endogenous flora triggering ASCA and to evaluate a possible link between *C. albicans* colonization and CD.

Here, we have used this collection to define the prevalence of digestive commensal carriage of *Candida* spp. and to analyze, using MLST, the potential relationships that exist between *C. albicans* isolates that are carried by a single individual or by different individuals within the same family. Our results show that the rate of cross-transmission within families was high. Furthermore, we show that isolates within an individual or a family can differ through microevolution of one or several of the sequenced MLST loci. Importantly, these microevolutions are always associated with a loss of heterozygosity (LOH), suggesting a predominant contribution of mitotic recombination events during the evolution of commensal isolates.

MATERIALS AND METHODS

Population and strains. *Candida* sp. strains were collected in the framework of a case-control study aimed at analyzing the correlation between *Candida* carriage in the digestive tract and the presence of ASCA and other genetic and environmental factors associated with CD (12, 44). This study included 177 subjects from 25 families (i.e., parents and first and second generations of children) from northern Belgium. Each family included one to seven individuals with CD. There were 79 subjects with CD and 98 unaffected family members. Fifty-seven subjects from 10 families with no cases of CD but living in the same geographical area were used as controls. A specific mycological sampling procedure was included in the study. A mouth swab and a stool sample were obtained from each subject and cultured extemporaneously on CHROMagar Candida medium (Bio-Mérieux, Marcy-l'Etoile, France). After growth, for each plate, all colonies exhibiting different colors were further identified either by Bichro-Latex-Albicans (Fumouze, Levallois, France) for *C. albicans* or by the API 32C system (Bio-Mérieux, Marcy-l'Etoile, France) for other species. For each sample, one *Candida* sp. clone was stored at −80°C in Cryo-bille tubes (AES, Combourg, France). *C. albicans* isolates were then subcultured for DNA extraction.

MLST for *Candida albicans*. *C. albicans* isolates were typed using the original MLST scheme described previously by Bougnoux et al. (5). Methods for DNA preparation, amplification, and determination of nucleotide sequences have been presented in detail previously (5). Sequence analysis was performed using ABI PRISM SeqScape software, version 2.0 (Applied Biosystems, CA). Each strain is characterized by a DST resulting from the combination of the genotypes obtained at the six following loci: *ACC1*, *VPS13*, *GLN4*, *ADP1*, *RPN2*, and *SYA1*. Genetic relatedness between the investigated strains was evaluated by constructing an unweighted-pair group method with arithmetic averages dendrogram using START software (http://outbreak.ceid.ox.ac.uk), which compares genotypic profiles (14). In addition, each strain was introduced into the original MLST online database (http://calbicans.mlst.net), which allowed us to compare the strains to those previously typed by this method, and deposited in the database (4).

Sequencing of haplotypes. PCR-generated DNA fragments corresponding to loci where microevolutions had been detected through MLST were cloned into the pCR2.1-TOPO vector (Invitrogen Life Technologies, France). Inserts in one to eight pCR2.1-TOPO derivatives obtained for each individual cloning procedure were amplified by PCR, and the resulting products were directly sequenced for the identification of haplotypes. Sequence analysis was performed as described above.

Identification of *C. albicans* genetic groups. The genetic group of each isolate was defined through a comparison of the MLST profile of the isolate with those previously determined for 24 *C. albicans* reference strains from group I, 14 strains from group II, 15 strains from group III, 10 strains from group SA, 9

strains from group E, and 5 outliers, as determined using DNA fingerprinting with the Ca3 probe (30). Each strain was assigned to one of the genetic groups that constitute the *C. albicans* population.

Determination of *C. albicans* serotype. Serotypes A and B were determined by using the Iatron agglutination kit (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) according to the manufacturer's instructions. For strains with equivocal agglutination patterns, serotypes were confirmed by phospholipomannan polyacrylamide gel serotype-specific migration patterns in Western blots using anti-β-mannoside antibodies (42).

RESULTS

Prevalence of carriage of *Candida* spp. and *C. albicans* in the digestive tract of patients with Crohn's disease and healthy subjects. This study included 177 subjects from 25 families (i.e., parents and first and second generations of children) from northern Belgium, all including one to seven individuals with CD, and 57 subjects from 10 families with no cases of CD but living in the same geographical area. For each subject, *Candida* strains were recovered from the mouth and feces. One hundred twenty-five (53.4%) of the 234 subjects were colonized by one or several species of *Candida* (111 subjects [88%] were colonized by only one species). Fifty-eight subjects (25%) were colonized at only one of the two sites studied (mouth [30 subjects] and feces [28 subjects]), whereas 67 subjects (29%) were colonized at both sites. *C. albicans* was identified in 88% of the *Candida* carriers (109/125), *C. glabrata* was identified in 9% of the carriers (12/125), *C. parapsilosis* was identified in 4% of the carriers (5/125), and another *Candida* species (including *C. norvegensis*, *C. sake*, *C. utilis*, *C. tropicalis*, and *C. lusitaniae*) was identified in 7% of the carriers (9/125). *C. albicans* carriers were identified in all of the 35 families except 2 (one control family and one family with subjects with CD). Overall, the rate of digestive carriage of *C. albicans* was 46.5%.

Prevalence rates of digestive carriage of *Candida* spp. and *C. albicans* in the two different types of families are presented in Table 1. There was a trend towards an increased prevalence of carriage of *Candida* spp. and of *C. albicans* in the CD families (prevalence ratios of 1.17 and 1.21, respectively). However, this trend was not found to be statistically significant. Prevalence was not significantly different in individuals with CD (49.3%; 39/79) and in healthy members of their families (48%; 47/98), and it was not significant in the 57 members of the 10 control families (40.3%; 23/57) (Table 1). Similarly, prevalence of carriage of *Candida* spp. and of *C. albicans* was not found to be significantly different between ASCA-positive and ASCA-negative CD subjects (data not shown).

MLST analysis of *C. albicans* isolates. Taken together, 166 *C. albicans* isolates were obtained: 30 were obtained from subjects colonized in the oral cavity only, 24 were obtained from subjects colonized in the intestinal tract only, and 112 (56 pairs) were obtained from subjects colonized concomitantly in the oral cavity and in the intestinal tract. MLST analysis focused on 82 *C. albicans* isolates obtained from 56 different carriers distributed within 19 families (26 carriers were colonized at both sites). As shown in Table 2, 46 different DSTs were identified: 26 (57%) corresponded to a single isolate, whereas the remaining 20 were shared by multiple isolates (1 by nine isolates, 2 by four isolates, 5 by three isolates, and 12 by two isolates). Two DSTs included isolates obtained from subjects from different families: DST 61 was shared by six subjects distributed between families F1 (one subject), F6 (one subject), F12 (two subjects), and F16 (two subjects), and DST 95 was shared by three subjects distributed between families F15 (one subject) and F17 (two subjects) (Table 2). The 18 remaining DSTs shared by several isolates were specific to either one subject or one family (Table 2 and Fig. 1).

TABLE 1. Prevalence of digestive carriage of *Candida* spp. and *C. albicans* among the 234 subjects from the 35 families studied

Type of family	No. of subjects	Prevalence (%) of digestive carriage of:	
		Candida spp.	*C. albicans*
Family with cases of CD (n = 25)	177	55.3	48.5
Subjects with CD	79	57	49.3
Healthy subjects	98	54	48
Family control (n = 10)	57	47.3	40.3
Total	234	53.4	46.5

A mixed dendrogram (not shown) was generated using the MLST data from reference strains and the 82 isolates from the 19 families mentioned above. Since the clades of the reference strains were known, the 82 isolates could be assigned, on the basis of their coclustering characteristics, to one of six *C. albicans* clades that have been previously defined: I, II, III, III′, SA, and E (3, 29, 30). Clades III and III′ have been defined on the basis of MLST data and include strains that are found within clade III when Ca3 fingerprinting was used (Bougnoux et al., Abstr. 7th ASM Conf. Candida Candidiasis 2004). Twelve isolates (15%) did not group into any of the six clades, 19 were grouped into clade I (23%), 4 were grouped into clade II (5%), 11 were grouped into clade III (13%), 5 were grouped into clade III′ (6%), 2 were grouped into clade SA (2.5%), and 29 were grouped into clade E (35%). Figure 2 illustrates the clustering of the 82 isolates in the different clades. In 11 (79%) of the 15 families that included at least two carriers of *C. albicans*, isolates belonged to different genetic groups (two to four different groups) (Table 2 and Fig. 2). In the 26 carriers positive by mouth and feces, only 2 (8%) had isolates from two different genetic groups (subject 1 in family F1 [isolates C1 from group III′ and C2 from group E] and subject 30 in family F12 [isolates C68 from group III and C69 from group E]) (Table 2 and Fig. 1).

The 82 isolates were also subjected to serotyping. Seventy-four isolates were serotype A, and 8 isolates were serotype B. Serotype A isolates were present in all clades (Table 2). Serotype B isolates were found only in clades I and II. However, this may result from the small size of the sample.

Analysis of familial *C. albicans* isolates. In 15 out of the 19 families, *C. albicans* isolates originated from different members. In these families, 22 (43%) of the 52 *C. albicans* carriers had one isolate with a DST identical to that of an isolate from another member of the same family, suggesting intrafamilial transmission. Indeed, in 17 instances, the subjects that shared *C. albicans* strains with the same DST lived in the same zip code area, while in five other instances, they lived in a contiguous zip code area (subjects 5 and 6 from family F3 lived in zip code area 3800 and 3806, and subjects 15, 17, and 19 from

TABLE 2. Characteristics of *C. albicans* isolates from the 56 carriers studied

Subject	CD	Area[a]	Family	Isolate	Origin	Genotype						DST	Clade[b]	Serotype
						CaACC1	*CaVPS13*	*CaGLN4*	*CaADP1*	*CaRPN2*	*CaSYA1*			
1	Yes	2260	F1	C1	Mouth	2	33	13	6	25	32	79	III′	A
				C2	Feces	13	60	10	22	8	11	61	E	A
2	No	2490	F2	C3	Mouth	13	5	10	22	8	11	57	E	A
				C4	Feces	13	5	10	22	8	11	57	E	A
3	No	2490	F2	C5	Mouth	2	5	10	22	8	11	372	E	A
				C6	Feces	13	5	10	22	8	11	57	E	A
4	No	2490	F2	C7	Feces	2	60	10	22	27	11	94	E	A
5	Yes	3800	F3	C11	Feces	10	50	11	10	13	30	47	III	A
6	Yes	3806	F3	C12	Mouth	10	50	11	10	13	30	47	III	A
				C13	Feces	10	50	11	10	13	30	47	III	A
7	No	3320	F4	C15	Feces	5	11	8	15	4	4	112	SA	A
8	No	3300	F4	C16	Mouth	6	30	9	23	32	35	121	NC	A
9	Yes	3473	F4	C17	Mouth	20	26	3	10	2	14	98	NC	A
				C18	Feces	20	26	3	22	2	14	99	NC	A
10	Yes	3473	F4	C21	Mouth	20	26	3	22	2	14	99	NC	A
				C22	Feces	20	26	3	22	2	14	99	NC	A
11	No	8480	F6	C25	Mouth	10	25	9	28	20	30	147	NC	A
12	No	8400	F6	C28	Mouth	13	60	10	22	8	11	61	E	A
				C29	Feces	13	60	10	22	8	11	61	E	A
13	Yes	8810	F6	C30	Mouth	2	60	8	22	8	6	96	E	A
				C31	Feces	2	60	8	22	8	6	96	E	A
14	Yes	8400	F6	C32	Mouth	6	7	11	13	14	4	126	I	A
15	Yes	8650	F6	C34	Mouth	7	7	11	13	14	4	133	I	B
16	No	8610	F6	C35	Mouth	5	5	9	25	14	18	118	NC	A
				C36	Feces	5	5	9	25	14	18	118	NC	A
17	No	8670	F6	C37	Mouth	7	7	11	13	14	4	133	I	B
				C38	Feces	7	7	11	13	14	4	133	I	B
18	No	8610	F6	C39	Mouth	8	5	8	14	8	8	142	II	A
				C40	Feces	8	5	8	14	8	8	142	II	A
19	Yes	8810	F6	M42	Feces	7	7	11	13	14	4	133	I	B
20	No	3850	F8	C44	Mouth	17	60	10	22	8	11	67	E	A
				C45	Feces	17	60	10	22	8	11	67	E	A
21	Yes	3570	F8	C46	Mouth	4	50	11	10	13	33	148	III	A
				C47	Feces	4	50	11	10	13	33	148	III	A
22	No	3770	F9	C54	Mouth	7	7	9	13	14	4	19	I	A
				C55	Feces	7	7	9	13	14	4	19	I	A
23	Yes	1652	F10	C118	Mouth	6	21	11	13	14	4	39	I	B
				C119	Feces	6	21	11	13	14	4	39	I	B
24	No	1652	F10	C120	Mouth	6	21	11	13	14	4	39	I	B
25	No	3570	F11	C59	Feces	13	5	1	22	8	11	56	E	A
26	Yes	3520	F11	C60	Feces	7	7	15	13	14	4	151	I	A
27	No	3570	F11	C63	Feces	13	5	1	22	8	11	56	E	A
28	Yes	3920	F12	C64	Mouth	10	12	11	22	10	9	41	III	A
				C65	Feces	10	12	11	22	10	9	41	III	A
29	No	3920	F12	C66	Mouth	2	4	13	6	22	12	85	III′	A
				C67	Feces	2	4	13	6	22	12	85	III′	A
30	Yes	3920	F12	C68	Mouth	10	12	11	22	10	6	314	III	A
				C69	Feces	13	44	13	27	8	6	54	E	A
31	No	3920	F12	C70	Mouth	13	60	10	22	8	11	61	E	A
				C71	Feces	13	60	10	22	8	11	61	E	A
32	Yes	3920	F12	C74	Mouth	13	60	10	22	8	11	61	E	A
33	No	3920	F12	C78	Mouth	14	114	11	22	10	9	373	III	A
				C79	Feces	15	12	11	22	10	9	313	III	A
34	Yes	3300	F13	C97	Feces	7	39	2	13	28	4	128	NC	A
35	Yes	8610	F14	C111	Mouth	2	47	13	6	7	12	86	III′	A
36	No	8690	F14	C114	Feces	7	40	14	13	14	4	130	I	A
37	Yes	8310	F15	C131	Mouth	2	60	10	22	8	11	95	E	A
38	No	8310	F15	C132	Mouth	6	40	11	13	14	4	122	I	A
				C133	Feces	6	40	11	13	14	4	122	I	A
39	No	8750	F15	C134	Feces	2	60	1	22	8	11	92	E	A
				C135	Mouth	2	60	1	22	8	11	92	E	A
40	Yes	8000	F15	C142	Mouth	2	60	1	39	8	11	315	E	A
41	Yes	2580	F16	C152	Mouth	13	60	10	22	8	11	61	E	A
				C153	Feces	13	60	10	22	8	11	61	E	A
42	No	2580	F16	C155	Mouth	13	60	10	22	8	11	61	E	A
43	No	3560	F16	C156	Mouth	2	33	13	6	22	12	78	III′	A

Continued on following page

TABLE 2—*Continued*

Subject	CD	Area[a]	Family	Isolate	Origin	Genotype						DST	Clade[b]	Serotype
						CaACC1	*CaVPS13*	*CaGLN4*	*CaADP1*	*CaRPN2*	*CaSYA1*			
44	Yes	2630	F17	C161	Mouth	2	37	1	24	2	16	84	NC	A
				C162	Feces	7	37	1	24	2	16	150	NC	A
45	Yes	9700	F17	C163	Mouth	2	60	10	22	8	11	95	E	A
				C164	Feces	2	60	10	22	8	11	95	E	A
46	Yes	2630	F17	C166	Mouth	4	56	8	30	19	8	149	II	B
				C167	Feces	4	56	8	30	19	8	149	II	B
47	No	9700	F17	C168	Mouth	2	60	10	22	8	11	95	E	A
48	No	3461	S1	C91	Mouth	10	48	11	10	13	9	46	III	A
49	No	8970	S3	C84	Mouth	7	7	14	13	14	4	135	I	A
50	No	8970	S3	C87	Mouth	2	35	1	24	2	31	83	NC	A
51	No	8970	S3	C90	Mouth	13	60	8	22	29	11	62	E	A
52	No	2220	T1	C157	Mouth	5	4	10	15	3	30	116	SA	A
53	No	2220	T1	C160	Feces	6	3	9	13	14	4	119	I	A
54	No	3550	T2	C121	Mouth	6	7	11	12	14	4	30	I	A
55	No	3550	T2	C122	Feces	6	7	11	12	14	4	30	I	A
56	No	3550	T2	C124	Mouth	6	7	11	12	14	4	30	I	A

[a] Area is indicated by the zip code of the town in which the subject lived.
[b] Isolates were assigned to one of the six *C. albicans* clades. NC, no clade.

family F6 lived in zip code areas 8650, 8657, and 8810) (Table 2 and Fig. 1), thus suggesting likely frequent encounters.

Family F12 provides a good illustration of the situations that were observed in this study (Table 2 and Fig. 2). Here, six *C. albicans* carriers were studied (subjects 28 to 33), five of which were carriers at both sites. Of these carriers, three carried isolates with identical DSTs at both sites (subjects 28, 29, and 31). Two carriers had isolates with identical DSTs (subjects 31 and 32). Three carriers had isolates with DSTs that differed by one or two loci only (subjects 28, 30, and 33). Subject 33 was of particular interest, since the subject carries two strains that differ at two loci only. These data suggested that in this family, colonization by a single isolate at both sites was frequent, that colonization could be associated with microevolution as indicated by the occurrence of strains with closely related DSTs, and that intrafamilial transmission was also frequent.

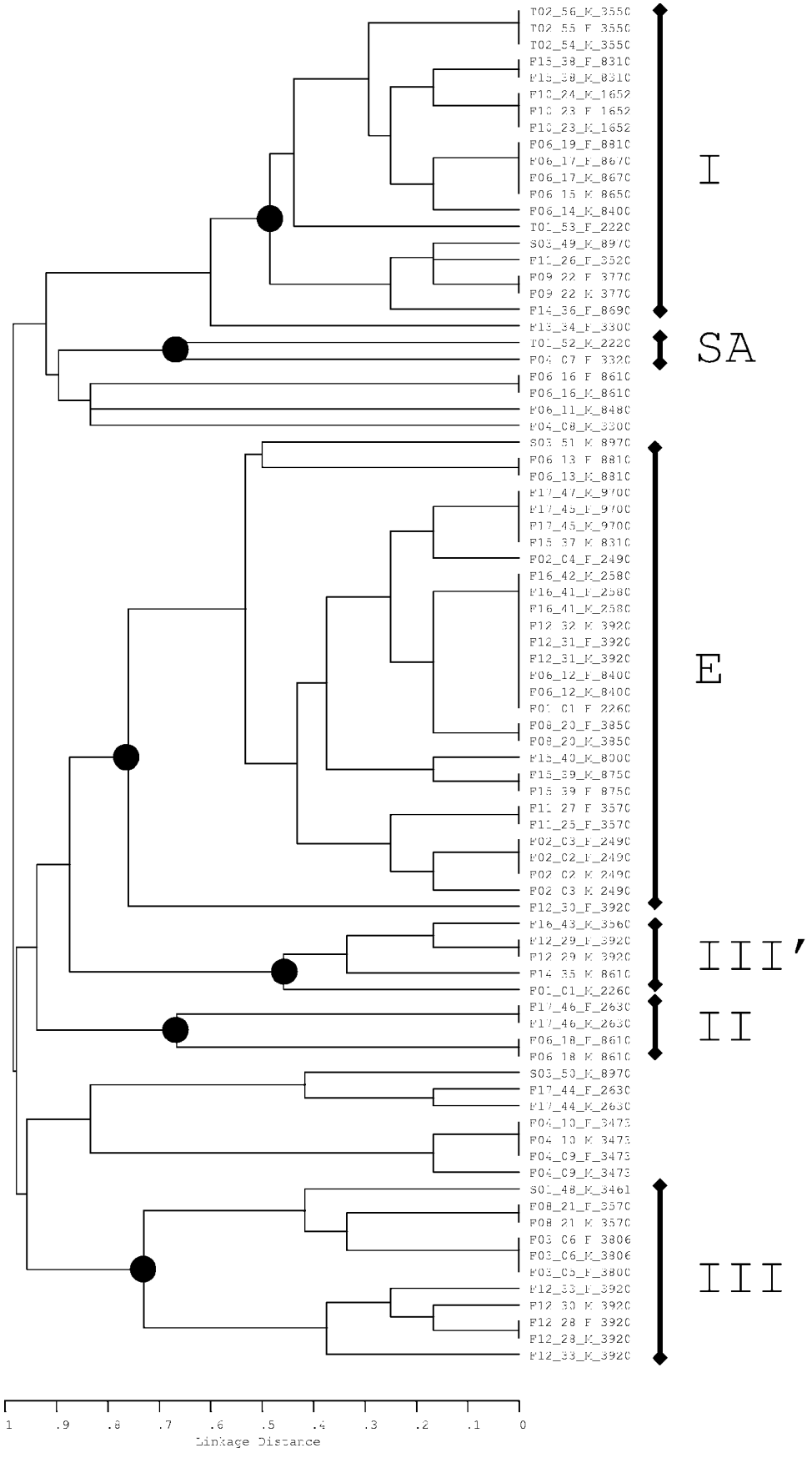
Taken together, the results presented in Table 2 and Fig. 2 show 20 carriers with isolates with identical DSTs obtained from both sites (77% of the carriers with isolates obtained at both sites), 11 examples where two or more individuals in one family carry an isolate with an identical DST (73% of the families with more than one *C. albicans* carrier), 4 examples where one individual carries isolates with DSTs that differ by one or two loci only (67% of the cases where a carrier harbors isolates with different DSTs), and 4 examples where two or more individuals within the same family carry isolates with DSTs that differ by one or two loci only (27% of the families with more than one *C. albicans* carrier). For each family, carriers with identical or microevolved DSTs always resided within close zip code areas. These data suggest that the trend observed in family F12, i.e., a high frequency of colonization by isolates of identical or closely related DSTs at both sites and of intrafamilial transmission, is common within the samples we have studied.

Characterization of the microevolutions at the MLST loci. Of particular interest to us was the identification of isolates that differed at one or two of the MLST loci within an individual or a family, since these isolates may provide a hint as to the molecular mechanisms that are involved in the dynamics of the genome during *C. albicans* commensalism. We therefore investigated the nature of the microevolved genotypes within four pairs of strains obtained from a single individual (C5 [DST 372] and C6 [DST 57] from subject 3 in family F2, differing at the *ACC1* locus; C17 [DST 98] and C18 [DST 99] from subject 9 in family F4, differing at the *ADP1* locus; C78 [DST 373] and C79 [DST 313] from subject 33 in family F12, differing at the *ACC1* and *VPS13* loci; and C161 [DST 84] and C162 [DST 150] from subject 44 in family F17, differing at the *ACC1* locus).

Sequence analysis of variant genotypes within each of these four pairs of isolates showed that variations between isolates resulted from a LOH at one or several of the polymorphic sites of one or two loci (Fig. 3A). In two instances, the differences between the two genotypes within a locus were limited to a single LOH (C5 versus C6, *AAC1*, nucleotide 29, A/T versus T/T; C18 versus C17, *ADP1*, nucleotide 125, A/G versus G/G) that may result from either a point mutation or a mitotic recombination event. In the three other instances, the divergence resulted from two or three LOHs that were never separated by a heterozygous polymorphic site (Fig. 3A).

For the four microevolved loci (*ACC1* locus for subjects 3, 33, and 44 and *VPS13* for subject 33), the PCR products that

FIG. 1. Dendrogram of the genetic relationships among 82 isolates of *C. albicans* based on MLST data. The dendrogram was constructed by using the unweighted-pair group method with arithmetic averages using the START software, which compares genotypic profiles (14). Strains were assigned to clades (I, II, III, III′, E, and SA) on the basis of their coclustering with a set of reference *C. albicans* strains that had been previously typed using the Ca3 probe and MLST. Strains are designated according to the following nomenclature: family_subject_origin_number_zip code. For origin, M indicates mouth and F indicates feces. The linkage distance is indicated at the bottom.


T02_56_M_3550
T02_55_F_3550
T02_54_M_3550
F15_38_F_8310
F15_38_M_8310
F10_24_M_1652
F10_23_F_1652
F10_23_M_1652
F06_19_F_8810
F06_17_F_8670
F06_17_M_8670
F06_15_M_8650
F06_14_M_8400
T01_53_F_2220
S03_49_M_8970
F11_26_F_3520
F09_22_F_3770
F09_22_M_3770
F14_36_F_8690
F13_34_F_3300
T01_52_M_2220
F04_07_F_3320
F06_16_F_8610
F06_16_M_8610
F06_11_M_8480
F04_08_M_3300
S03_51_M_8970
F06_13_F_8810
F06_13_M_8810
F17_47_M_9700
F17_45_F_9700
F17_45_M_9700
F15_37_M_8310
F02_04_F_2490
F16_42_M_2580
F16_41_F_2580
F16_41_M_2580
F12_32_M_3920
F12_31_F_3920
F12_31_M_3920
F06_12_F_8400
F06_12_M_8400
F01_01_F_2260
F08_20_F_3850
F08_20_M_3850
F15_40_M_8000
F15_39_M_8750
F15_39_F_8750
F11_27_F_3570
F11_25_F_3570
F02_03_F_2490
F02_02_F_2490
F02_02_M_2490
F02_03_M_2490
F12_30_F_3920
F16_43_M_3560
F12_29_F_3920
F12_29_M_3920
F14_35_M_8610
F01_01_M_2260
F17_46_F_2630
F17_46_M_2630
F06_18_F_8610
F06_18_M_8610
S03_50_M_8970
F17_44_F_2630
F17_44_M_2630
F04_10_F_3473
F04_10_M_3473
F04_09_F_3473
F04_09_M_3473
S01_48_M_3461
F08_21_F_3570
F08_21_M_3570
F03_06_F_3806
F03_06_M_3806
F03_05_F_3800
F12_33_F_3920
F12_30_M_3920
F12_28_F_3920
F12_28_M_3920
I
SA
E
III′
II
III
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
Linkage Distance

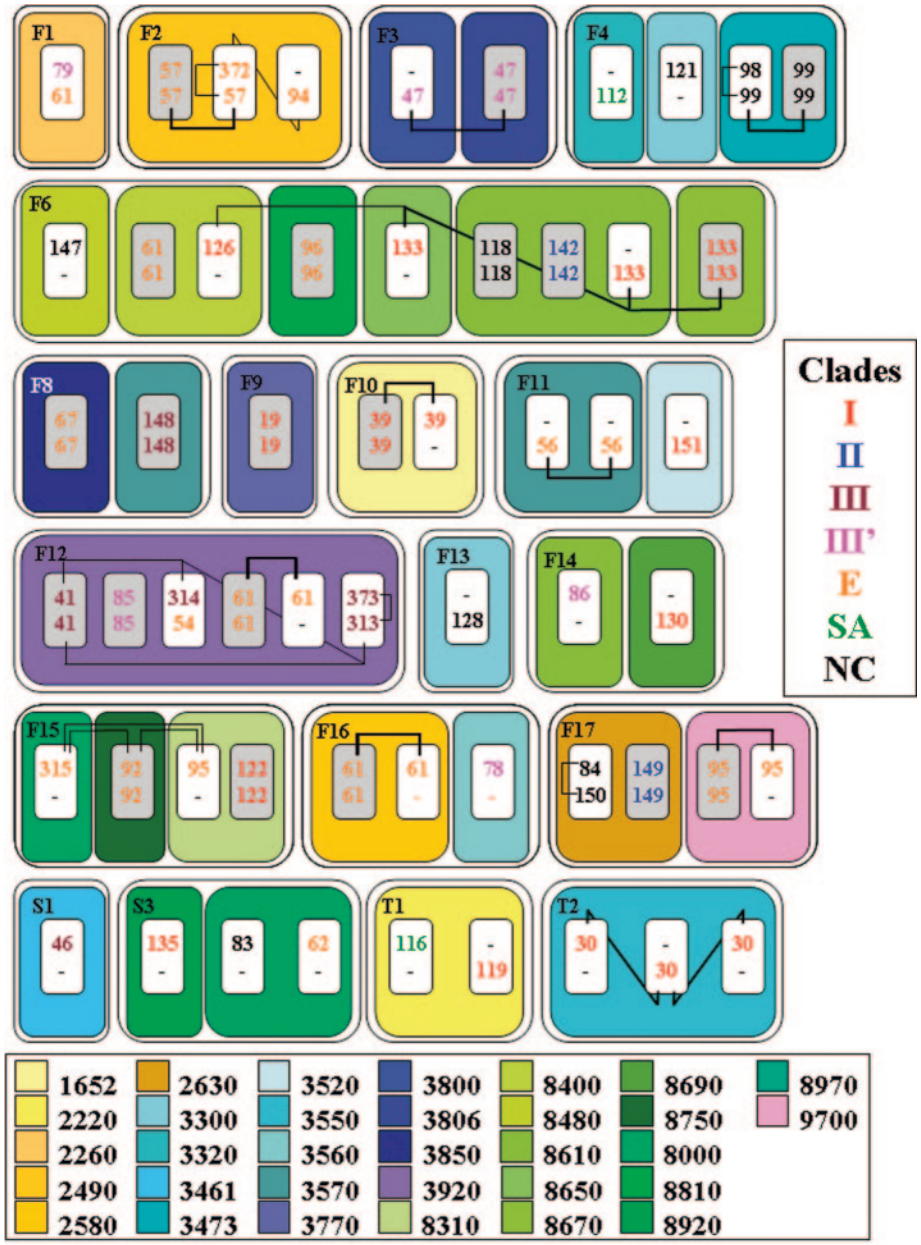


FIG. 2. Graphical representation of *C. albicans* familial carriage and genetic relationships among *C. albicans* strains. DSTs and clades of *C. albicans* strains carried at the oral (top) or fecal (bottom) site, zip code area of residence, and family are represented for each carrier whose isolates have been typed by MLST. Zip codes are color coded in order to indicate proximity (e.g., all zip codes between 8310 and 8970 are color coded in green). Subjects with isolates with identical DSTs at both sites are shaded in gray. Strains with identical DSTs and that are shared by two or more subjects are linked by a thick line, while closely related strains of different DSTs (see the text) are linked by a thin line.

had been sequenced by MLST were subcloned, and inserts in eight plasmids for each product were sequenced in order to define the haplotypes that when combined would yield the genotype observed by MLST. Analysis of the haplotypes in each of the three pairs of isolates showed that a first haplotype was shared by both isolates, while the other haplotypes differed between the two strains at the positions corresponding to the LOH observed by MLST (data not shown). Because LOH always occurred at the polymorphic sites identified within a large collection of strains by MLST and the distance between the sites of LOH was large (20 to 360 bp), it is likely that these LOH events reflect mitotic recombination events between complementary chromosomes.

We also investigated four pairs of strains obtained from two individuals within the same family (C32 [DST 126] and C34 [DST 133] from subjects 14 and 15 in family F6, differing at the *ACC1* locus; C64 [DST 41] and C68 [DST 314] from subjects 28 and 30 in family F12, differing at the *SYA1* locus; C64 [DST 41] and C79 [DST 313] from subjects 28 and 33 in family F12, differing at the *ACC1* locus; and C135 [DST 92] and C142 [DST 312] from subjects 39 and 40 in family F15, differing at the *ADP1* locus). The divergence always resulted from an LOH

A

ACC1

N° subject	Family	N° isolate	locus state	Genotype	Clade	Position of polymorphic nucleotide sites						
						8	29	119	211	281	317	392
3	F2	C6	Het	13	Ea	G	**A/T**	G	A	C	C	C
		C5	Hom	2	Ea	G	**T**	G	A	C	C	C
33	F12	C78	Het	14	III	**A/G**	**A/T**	G	A	C	C	**C/T**
		C79	Hom	15	III	**A**	**T**	G	A	C	C	**T**
44	F17	C162	Het	2	NC	G	T	G	**C/A**	C	**C/T**	C
		C161	Hom	7	NC	G	T	G	**A**	C	**C**	C

VPS13

N° subject	Family	N° isolate	locus state	Genotype	Clade	33	43	49	107	134	136	212	217	241	281	282	322	334	370	375
33	F12	C78	Het	114	III	G	C	C	A	T	G	**A/G**	**C/T**	**A/G**	G	**A/G**	T	**A/G**	**A/T**	C
		C79	Het	12	III	G	C	C	A	T	G	**A/G**	**T**	**G**	G	**A/G**	T	**A/G**	**A/T**	C

ADP1

N° subject	Family	N° isolate	Locus State	Genotype	Clade	35	40	46	62	109	125	166	205	215	225	232	263	282	352	395
9	F4	C18	Het	22	NC	A	**C/T**	**C/T**	C	**A/G**	**A/G**	**A/G**	**A/G**	G	**A/T**	**C/T**	A	G	A	A
		C17	Het	10	NC	A	**C/T**	**C/T**	C	**A/G**	**G**	**A/G**	**A/G**	G	**A/T**	**C/T**	A	G	A	A

B

ACC1

N° subject	Family	N° isolate	locus state	Genotype	Clade	8	29	119	211	281	317	392
14	F6	C32	Het	6	I	G	T	G	**C/A**	C	**C/T**	C
15		C34	Hom	7	I	G	T	G	**C**	C	**T**	C
28	F12	C64	Het	10	III	**A/G**	T	G	A	**C/T**	C	**C/T**
33		C79	Hom	15	III	**A**	T	G	A	**C**	C	**T**

SYA1

N° subject	Family	N° isolate	locus state	Genotype	Clade	1	25	32	37	61	100	142	160	185	307	324	351
28	F12	C64	Het	9	III	T	**A/C**	A	A	**A/G**	**C/T**	**A/G**	**C/T**	G	C	G	**C/T**
30		C68	Hom	6	III	T	**C**	A	A	**A**	**T**	**G**	**C**	G	C	G	**T**

ADP1

N° subject	Family	N° isolate	Locus State	Genotype	Clade	35	40	46	62	109	125	166	205	215	225	232	263	282	352	395
40	F15	C142	Het	39	Ea	A	**C/T**	**C/T**	C	**A/G**	**A/G**	**A/G**	**A/G**	G	**A/T**	**C/T**	A	G	A	A
39		C135	Het	22	Ea	A	**C/T**	**C/T**	C	**A/G**	**A/G**	**A/G**	**G**	G	**T**	**C**	A	G	A	A

FIG. 3. Characteristics of the variant genotypes between pairs of closely related *C. albicans* isolates. For each pair of isolates, the subjects, family, isolate numbers, and clade are indicated along with the sequence at the polymorphic sites within the microevolved locus. Two statuses can be observed at the investigated loci: a heterozygous state (Het) where at least one of the polymorphic sites is heterozygous (boldface type) and a homozygous state where all polymorphic sites are homozygous. Sites where a loss of heterozygosity has occurred between the two related isolates are shown in underlined boldface type. Panel A presents the data obtained for six pairs of isolates, each obtained from a single subject, while panel B presents the data obtained for four pairs of isolates obtained, each from two subjects within the same family.

at one locus with the replacement of two, three, or six heterozygous nucleotide sites by homozygous sites (Fig. 3B). Analysis of the haplotypes in two of the four pairs of isolates (C32/C34 and C64/C68) showed a pattern similar to that observed in the pairs described above, i.e., one haplotype shared by the two strains and two haplotypes each specific for a strain and differing by mutations corresponding to the LOH (data not shown). Again, LOH events were never separated by a heterozygous position and were located at polymorphic sites, indicating that they were likely to result from a mitotic recombination event.

DISCUSSION

An aim of this study was to evaluate the prevalence, diversity, and genetic relationships among *C. albicans* isolates recovered during natural colonization of the digestive tract of humans. For this purpose, we have screened oral and fecal samples from 234 subjects within 35 different families for *Candida* spp. The observed prevalence of commensal carriage (oral and/or fecal) of *Candida* spp. was 53.4%, indicating that digestive carriage of *Candida* spp. was very common in our samples. This prevalence is high in comparison with the data from the literature that indicate oral and anorectal carriage with a median prevalence of 24% and 27%, respectively (23). The concomitant analysis of two digestive sites (oral and fecal) used here is likely to allow a better evaluation of the digestive carriage of *Candida* spp. from healthy individuals and may explain our observation. Discrepancies between our results and those from other studies may also reflect differences in sampling procedures and thresholds used to distinguish colonized and uncolonized subjects. Our results also showed that few *Candida* species were identified in the digestive tract. A total of 91% of the carriers were colonized by only one *Candida* species, with 88% being colonized by *C. albicans*. *C. glabrata* was the second most frequently isolated species, colonizing 9% of the carriers. Because of the large number of subjects studied, our data are particularly valuable and emphasize the predominant role of *C. albicans* in the normal digestive flora of humans (87.8% of all isolates identified). This contrasts with

the observations that have been previously made in hospitalized patients. There, the epidemiology of digestive carriage of *Candida* spp. is strikingly different, with non-*C. albicans* isolates representing ca. 40% to 60% of all *Candida* isolates (9, 34, 41).

One of the main characteristics of our study was that 25 out of the 35 families included members affected by CD: 79 (34%) of the 234 subjects suffered from CD. Our results do not indicate any significant difference in the prevalence of commensal *Candida* carriage between individuals with CD and healthy members of their families or members of the 10 control families. Similarly, the distribution of species isolated as well as the high prevalence of *C. albicans* were not significantly different in the two groups of families studied (control families and families with CD members). Therefore, cross-transmission that may be higher in families where members suffer from severe intestinal disorders seems to have a low impact on the prevalence of commensal *Candida* carriage.

Finally, carriage of *Candida* spp. and *C. albicans* was not significantly different between ASCA-positive and ASCA-negative CD subjects. However, we have independently found a difference between CD patients and their healthy relatives and healthy controls in their ability to mount ASCA and anti-*C. albicans* mannan antibody responses in relation to *C. albicans* colonization (A. Standaert-Vitse, T. Jouault, P. Vandewalle, C. Mille, M. Seddik, B. Sendid, J. M. Mallet, J. F. Colombel, and D. Poulain, Abstr. 13th United Eur. Gastroenterol. Wk., abstr. 159, 2005). This latter observation would confirm a possible link between tolerance to *C. albicans* and CD. However, the nature of the alteration in CD patients underlying this tolerance and its relation to the immune response remain to be established.

MLST was used to evaluate the diversity and genetic relationships between 82 *C. albicans* isolates recovered during natural colonization of the digestive tract of 56 subjects within 19 families. These isolates could be assigned to 46 unique DSTs, indicating significant redundancy within our sample (see below). Despite this redundancy, we observed DSTs that belonged to almost all of the major clades that have been defined through molecular typing using the Ca3 probe and/or MLST (3, 29, 30) (Bougnoux et al., Abstr. 7th ASM Conf. Candida Candidiasis 2004).

This suggests that the geographic specificity that has been proposed for the different clades (clades I, II, and III; clade E; and clade SA, enriched for isolates from North America, Europe, and South Africa, respectively) should be reconsidered. A similar conclusion has been reached recently by Tavanti et al. (39) following the typing of 416 *C. albicans* isolates by MLST. Because our panel of strains, that used by Tavanti et al. (39), and those used for Ca3 typing have their own geographic bias, the geographical specificity of clades will have to be reevaluated when the typing of a large and geographically unbiased collection of isolates is completed. This will be facilitated by the nature of MLST that permits archiving of data generated by different laboratories worldwide in a central web resource. Interestingly, however, isolates of clade E and clade I formed the majority of those characterized in this study. Overall, isolates of clades E and I represented 58.5% of all isolates, and DSTs belonging to clade E and I represented 46.8% of all DSTs. If intrafamilial transmission was taken into account by grouping isolates of identical or closely linked DSTs into a single class (see below), the predominance of clades E and I remained (53.7%). A similar distribution between clades was observed for CD patients and healthy subjects. Similarly, intrafamilial transmission was not associated with a specific clade, suggesting that isolates of the different clades did not differ in their ability to be transferred between family members or to colonize individuals with CD.

As mentioned above, a significant number of strains with identical DSTs were observed in our study (55 isolates corresponding to 20 DSTs). Colonization of different sites in an individual by the same isolate has been reported previously (17, 46). Therefore, it is not surprising that in 19 subjects, two isolates with identical DSTs were obtained, indicating colonization of the two sampled digestive sites by the same or very closely related *C. albicans* isolates. Additionally, we observed 10 instances where isolates with identical DSTs were shared by several individuals (two or three individuals) within a family. Interestingly, these individuals always lived in the same household or same zip code area or in contiguous zip code areas, strongly suggesting intrafamilial transmission through frequent encounters. Adding to this, we observed the occurrence of isolates that differed at one or two loci only and that were likely to have evolved from a common ancestor in several families (see below). These isolates could be harbored by a single individual (five instances) or by several individuals (two to four individuals) within the family (four instances). Here again, these latter individuals lived near each other. It is therefore likely that these observations reflect other events of intrafamilial transmission where microevolution has occurred in one individual and only one microevolved isolate has been transmitted or where the ancestor has been transmitted and microevolution has occurred in the recipient individual. It is worth stressing here that sampling was simultaneous for all members of a family, indicating that strains evolved in parallel but differently, depending on the host harboring them. These findings demonstrated that intrafamilial transmission is frequent and is a potential source of contamination of humans, which is in agreement with previously published data (16, 21).

The observation of strains that differed at only one or two of the sequenced loci is indicative of microevolutions within the digestive tract. The fact that these strains differed through microevolutions is supported by our analysis of the haplotypes at each of the differing loci in pairs of related strains. In all instances, microevolutions were due to an LOH at one or several of the polymorphic sites within the sequenced locus. These LOH events can be explained by point mutations at the polymorphic sites or by mitotic recombination events between the two original haplotypes, resulting in a full or partial loss of heterozygosity. Although point mutations might account for cases where only one polymorphic site has evolved, we strongly favor mitotic recombination events in the other cases, because the LOH events encompass several polymorphic sites (if not all) within the locus. This hypothesis is supported by previous studies that have identified LOH events as a source of microevolutions in *C. albicans* (10, 11). However, the extent of these LOH events remains to be investigated. In this regard, these closely related isolates provide an invaluable resource to analyze the dynamics of the *C. albicans* genome that occur naturally during commensalism in the digestive tract. As mentioned

above, the occurrence of isolates with identical DSTs or differing through LOH events was frequent in our study, and this may be explained by the familial nature of the sampling. While most typing studies of *C. albicans* populations have reported a striking diversity within the species (6, 25, 38), some previous studies that addressed confined environments with a high probability of transmission have shown a lower diversity but have not evaluated the genetic origin of this phenomenon (5, 20, 32, 36). On the basis of our data, it is likely that these samples also contained a significant proportion of microevolved *C. albicans* isolates that contributed to reduce the overall diversity of the sample.

In summary, data presented in this study indicate that commensal carriage of *Candida* spp. and more specifically *C. albicans* is similar in CD patients and healthy subjects. This establishes that the presence of ASCA is not solely influenced by the *Candida* load but probably results from an alteration of the immune response occurring in CD patients (Standaert-Vitse et al., Abstr. 13th United Eur. Gastroenterol. Wk. 2005). Independently, our data show that the digestive tract is an important site for commensalism and associated intrafamilial transmission as well as microevolutions of *C. albicans*. While it was suggested in a recent study that LOH at the mating-type-like locus could be the result of antifungal pressure (33), our data suggest that at least at other loci, and in the population studied, LOH is a phenomenon that occurs naturally in the natural history of *C. albicans* carriage. Future studies should aim at detailing the nature and molecular basis of these microevolutions and evaluating their impact on the fitness of *C. albicans* isolates in the context of the digestive tract.

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